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Research Article

A morphological and high throughput sequencing workflow to identify Australian ants (Hymenoptera, Formicidae): a new tool for biosecurity and biodiversity

Francesco Martoni[1], James Buxton[1], Kathryn S. Sparks[2], Tongda Li[1], Reannon L. Smith[1], Lea Rako[1], Mark J. Blacket[1]

1 *Agriculture Victoria Research, AgriBio Centre, Bundoora, VIC 3083, Australia*
2 *Department of Agriculture, Fisheries and Forestry, Canberra, ACT 2601, Australia*
Corresponding author: Francesco Martoni (Francesco.martoni@agriculture.vic.gov.au)

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Abstract

Ants are often the most ubiquitous and ecologically influential components of terrestrial systems, exhibiting an exceptionally high level of endemic diversity. Simultaneously, exotic ants can be amongst the most environmentally and economically devastating biological invaders. Distinguishing between exotic and related natives is essential for early detection and to reduce the chance of establishment, but at the species-level there remains a great deal of uncertainty among several of the most diverse, widespread and ecologically dominant genera. This taxonomic impediment negatively impacts the use of molecular techniques relying on reference databases, due to the poor state of ant DNA sequences in publicly available repositories, with many species not represented at all and others incorrectly identified. As a result, biosecurity screening for targeted exotic ants and assigning ant species-level identifications for biodiversity remain two separate areas of focus in Australia.

Here we propose a workflow for the identification of ants that enables simultaneous processing of large numbers of "bulk" ant samples each containing many individuals, increasing resolution for taxonomic assignment, and generating a curated database linked to voucher specimens. We use a non-destructive DNA extraction method and compare two high throughput sequencing (HTS) metabarcoding platforms – MiSeq (Illumina) and MinION (Oxford Nanopore Technologies) – processing up to 180 bulk mixed ant samples per run. This approach allowed for the acquisition of curated DNA sequences from voucher specimens morphologically examined by expert taxonomists. This work highlights the advantages and current limitations of DNA-based identifications, the needs for standardisation, as well as the importance of a taxonomy-based curation of DNA database for both biodiversity and biosecurity.

Key words: Entomological collections, metabarcoding, non-destructive, taxonomy

Introduction

Ants are a group of insects of great importance for biosecurity. Invasive ants have the potential to cause substantial damage to ecosystems worldwide, especially in the Asia-Pacific Region (Hoffmann et al. 2017; Vanderwoude et al. 2021; Xu et al. 2022). Invasive ant incursions have the potential to cause

significant economic losses to agriculture and livestock (Morrison et al. 1999; Wu et al. 2014; Prodhan et al. 2023), as in the case of the red imported fire ant, *Solenopsis invicta*, which has already caused an estimated US$ 6 billion losses in the USA (Teal et al. 1999; Lard et al. 2001). More generally, in Australia, invertebrate pests are estimated to cause losses of more than $300 million every year (Murray et al. 2013). These risks warrant important biosecurity measures for early detection and eradication of any exotic pests. With an estimated 7500 species, the Australian ant fauna is considered by some to be 'megadiverse', especially due to the variety of climates and ecological niches present, offering a variety of habitats for different ant groups (Andersen 2016). However, the diversity is extremely poorly documented for several major groups, with some researchers suggesting that genera such as *Monomorium* and *Tetramorium* could contain more than 750 and 500 undescribed species respectively in Australia alone (Sparks et al. 2014; Andersen 2016), with only 250 and 300 currently known described species worldwide (Bolton 1995). These estimates have yet to be validated and the best means of morphologically and molecularly delimiting species for these genera has been the source of some controversy (Andersen et al. 2013; Heterick and Majer 2018; Andersen et al. 2023).

During the past two decades, the DNA barcoding technique (Hebert et al. 2003) has been widely used for ant biodiversity assessments around the world, mostly targeting the subunit I of the *cytochrome oxidase* (COI) mitochondrial gene (e.g., Smith et al. 2005; Ng'endo et al. 2013; Schär et al. 2018). The utility of COI barcodes for ant species delimitation can vary widely depending on the genus or species group studied, with most consistently providing 1–3% difference between different species (Smith et al. 2005). However, in other cases, no COI variation can be observed between distinct species (Schär et al. 2018), while other taxa exhibit sensationally higher COI variation within a species than is generally expected (Ng'endo et al. 2013). The last major revision of an Australian ant genus (Heterick et al. 2017; 2019) suggested that COI alone may overestimate species diversity considerably. While this has been suggested also for other groups of invertebrates (Beebe 2018; Hupało et al. 2023), it has also been disputed for ants (e.g., Andersen et al. 2020).

Ideally, an integrative taxonomic approach that includes a morphological component, and multiple gene regions is considered a way to overcome the apparent shortcomings of COI barcode-based delimitation (e.g., Cheng et al. 2023), with COI data never the sole basis for delimitation but rather a tool to inform hypotheses (Fisher and Smith 2008; Ng'endo et al. 2013). However, biosecurity surveillance does not allow for the time and resources for a detailed integrative approach for each specimen, often dealing with large numbers of samples, each of which may represent an exotic invader. Narrowing down possible targets using COI barcoding approaches has been demonstrated to be an effective delimitation tool for several exotics and is considered generally effective at the genus level (Armstrong and Ball 2005; Smith et al. 2005; Fisher and Smith 2008). Unfortunately, while a relatively small number of expert taxonomists can provide precise identifications for most species that pose a threat to biosecurity, this activity is also extremely time consuming, and therefore often limited to determining presence/absence of a pest, without focusing on identification of native and endemic species of no biosecurity concern.

On the other hand, high throughput sequencing (HTS) techniques – such as metabarcoding – are ideally placed to process large volumes of samples, to

simultaneously provide identifications for both target and non-target taxa. For the past decade, most studies focusing on detection of invertebrates for biosecurity using metabarcoding have relied on short-read sequencing platforms such as Illumina MiSeq (Comtet et al. 2015; Batovska et al. 2016; Fletcher et al. 2017; Piper et al. 2019; Martoni et al. 2023 a,b). These works relied on the fact that short-read sequencing platforms have a generally much lower error rate compared to longer-read sequencers such as Oxford Nanopore Technologies (ONT) MinION (Piper et al. 2019). In the context of metabarcoding, where the DNA reads generated are generally used to determine the species diversity within 'bulk' samples, each consisting of many individuals, low error rate and high accuracy are of paramount importance to distinguish errors from real diversity (Benítez-Páez et al. 2016; Krehenwinkel et al. 2019; Piper et al. 2019).

However, long-read instruments could improve taxonomic resolution (Tedersoo et al. 2018; Heeger et al. 2018) by providing longer DNA sequences that are more informative, or even whole mitogenomes that could be used for phylogenetic studies, as it has been demonstrated for browsing ants (Prodhan et al. 2023). Long-read nanopore devices are becoming increasingly popular because of the lower cost and high portability (Menegon et al. 2017), as well as the constant improvements being made to their chemistry, which has lowered the error rate to reach a current accuracy of > 99.9%. Therefore, the lower initial economic investment required for purchasing the sequencer, as well as its portability, make MinION the ideal tool to conduct diagnostic identification at biosecurity borders (Piper et al. 2019). While MinION is widely used for a variety of methodologies aiming at species identification, including whole genome sequencing, metagenome sequencing and transcriptomics (Bronzato Badial et al. 2018; McNaughton et al. 2019; Franco-Sierra and Díaz-Nieto 2020; Oppenheim et al. 2020), DNA barcoding and metabarcoding remain the fastest approaches for species identification, as they are relatively analytically simple in not requiring sequence assembly prior to analysis of DNA sequences for species identification. Furthermore, MinION sequencing offers a fast, portable and cost-effective approach to analysing smaller batches of samples, making it ideal for border biosecurity detections and a promising diagnostic tool for the identification of invertebrate pests (Srivathsan et al. 2018, 2019, 2023; Abeynayake et al. 2021).

Irrespective of the sequencing platform used, however, the taxonomical assignment of specimens using HTS metabarcoding is often hindered by the limited availability and quality of DNA reference sequences on public databases. Piper and colleagues (2019) highlighted a number of practical issues arising from this. In fact, sequences may not only be unavailable for a high number of taxa, but the sequences that are present on public databases may include errors and mistakes, such as barcode sequences being insufficiently annotated (Porter and Hajibabaei 2018), annotated with the incorrect species name (Boykin et al. 2012; Shen et al. 2013; Mioduchowska et al. 2018; Galan et al. 2018; Bengtsson-Palme et al. 2016), or with multiple morpho-species assigned to the same DNA barcode, caused by specimen misidentifications or the existence of species complexes (Ashfaq et al. 2016). These issues highlight the importance of engaging taxonomic experts to ensure *a priori* identification of a specimen before submitting a reference barcode to a public database (Batovska et al. 2016; Collins and Cruickshank 2013).

The aim of this work was to focus on Australian ants, as a biosecurity model which requires dealing not only with a large number of samples, but also with

numerous native/endemic species that are often understudied or poorly represented in public sequence databases. Our focus was to develop a workflow capable of utilising and preserving specimens and DNA sequence data that is often filtered out from biosecurity procedures because it is not associated with the target exotic pests. Ultimately, this work had the aims of: i) developing an identification workflow combining high throughput molecular sequencing with morphological examinations and high-resolution photographs of voucher specimens; ii) testing the upscaling of sample volume using a short-read metabarcoding pipeline for the identification of bulk-trapped ants; iii) testing the upscaling of sample volume using a long-read in-field compatible metabarcoding pipeline for the identification of bulk trapped ants, targeting a longer, more informative genetic fragment.

Material and methods

Sample collection and preservation

A total of 180 bulk ant samples were collected at various Australian ports of entry in New South Wales and Western Australia, using baited traps or hand-collected from the ground. Insects were instantly killed with high grade ethanol (95–100%) before a preliminary morphological identification was performed by biosecurity diagnosticians at ports of entry, aiming to exclude the presence of exotic pests (Suppl. material 1). Samples were then shipped to the AgriBio research centre in Victoria, Australia, for further analysis. Upon receipt, non-destructive DNA extractions were performed on the samples (see below), and an identification number was applied (Suppl. material 1). The samples were then subdivided into two batches of 90 samples each for downstream analysis.

DNA extraction and amplification, and MiSeq Illumina library preparation

The DNA extraction protocol used a Dneasy Blood and Tissue kit (Qiagen, Germany) and it is based upon the protocol outlined in Martoni et al. (2019, 2021a). Briefly, ethanol was removed from the vials and the ants were submerged in a 9:1 solution of ATL buffer and Proteinase K and incubated at 56 °C overnight (approximately 17–18 hours). The overall ATL/Proteinase K volume would vary depending on the number of ants present in each vial, ranging from 500 µL (1–10 ants) to a few mL (>100 ants). Due to the nature of baited traps, the insects collected usually consisted of ants of similar sizes, hence not requiring a size-based splitting of the samples as presented elsewhere (Elbrecht et al. 2021; Martoni et al. 2023a, b). After incubation, samples were processed following the methods presented in Martoni et al. (2019), using a Dneasy Blood and Tissue kit (Qiagen, Germany).

Polymerase Chain Reaction (PCR) amplification was conducted using primers modified by incorporating partial Illumina adapter sequences (in bold) into the primer pair fwhF2 (5'-**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**GGDACWGGWTGAACWGTWTAYCCHCC-3')- fwhR2n (5'-**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**GTRATWGCHCCDGCTARWACWGG-3') (Vamos et al. 2017), targeting a 205 bp amplicon (excluding primers and adapters) from 346 bp to 551 bp within the conventional COI barcode region. PCR was performed using the Bioline MyFi DNA Polymerase kit (Meridian Bioscience, Ohio, USA) using 2.5 µL of DNA

template, 14.7 µL Bovine Serum Albumin (New England Biolabs, Massachusetts, USA), 5 µL of 5x Bioline MyFi reaction buffer, 0.8 µL MyFi DNA polymerase, and 1 µL each for the two primers (10 mM) in a 25 µL final volume. The PCR was run with the following cycling conditions: an initial 5-minute denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 7 minutes. PCR amplification was verified on a 1% w/v agarose gel.

Three separate MiSeq libraries were prepared for sequencing. The first MiSeq metabarcoding library (Run1) was prepared using the first 90 samples (ID: 1–90) while a second library (Run2) was prepared using the remaining 90 samples (ID: 91–180). Finally, a third library (Run3) was prepared by combining the first two libraries, therefore including all 180 samples.

For each library, PCR amplicons were diluted 10 times and used as template for a second round of real time PCR (rtPCR) to attach the remainder of the Illumina sequencing adapters and unique dual indexes to each sample, using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA). PCR conditions were an initial denaturation of 30 seconds at 98 °C followed by 7 cycles of denaturation at 98 °C for 10 seconds, annealing at 65 °C for 30 seconds and elongation at 72 °C for 30 seconds. Amplicons were purified and normalized using a SequalPrep Normalization Plate kit (Thermo Fisher Scientific, Massachusetts, USA). Each indexing rtPCR reaction (50 µL volume) contained 32.5 µL of ddH2O, 10 µL of 5 x Phusion HF Buffer, 1 µL of dNTP mix (10 mM), 1 µL of SYBR Green I Mix (Thermo Fisher Scientific, Massachusetts, USA) diluted 1/1000 in ddH2O, 0.5 µL Phusion DNA polymerase, 4 µL of sample-specific indexing primers (2.5 µM) and 1 µL of the diluted PCR product.

Library fragment size (amplicon + adapters + indexes) and absence of primer dimers was verified on an Agilent TapeStation (Agilent Technologies, California, USA) and all libraries were equimolarly pooled based on their concentrations as determined by Qubit dsDNA HS Fluorometric Quantification (ThermoFisher Scientific, Massachusetts, USA). As DNA concentrations in negative controls were too low to be measured, they were pooled at the same volume of the lowest concentration mock community library. The final pooled libraries (Runs 1–3) were then diluted to 7 pM, spiked with 15–25% PhiX (due to the expected low diversity of the library), and sequenced on the Illumina MiSeq platform using the V3 reagent kit (2 × 250 bp reads) (Illumina, California, USA).

Raw sequence reads were demultiplexed using bcl2fastq v2.2.0 allowing for no mismatches to the expected index combinations. The remaining quality control steps were performed using the software R v4.0.2 (R Core Team 2024) and the script available on GitHub (https://github.com/alexpiper/iMapPESTS/blob/master/local_metabarcoding.Rmd). Reads were trimmed of PCR primer sequences using BBDuK v38.9 (Bushnell et al. 2017). Sequence quality profiles were used to further filter reads with > 1 expected error (Edgar and Flyvbjerg 2015), or any ambiguous 'N' Bases, then remaining sequences were denoised using DADA2 v1.16 (Callahan et al. 2016) with the error model determined separately for each sequencing run. Following denoising, amplicon sequence variants (ASVs) inferred separately from each sequencing run were combined into a single table and any chimeric sequences removed de-novo using the "removeBimeraDenovo" function in DADA2. Retained ASVs were then checked for frame shifts and stop codons that commonly indicate pseudogenes (Roe and

Sperling 2007). Taxonomy was determined using BLASTn v2.11.0 with a minimum percentage identity of 97% and minimum alignment coverage of 95%. The ASVs that couldn't be accurately mapped were discarded using filtering functions contained in the phyloseq v1.36.0 (McMurdie and Holmes 2013) and tidyverse v1.3.1 (Wickham et al. 2019) R packages.

DNA analysis and MinION Nanopore extraction and amplification

The first 90 ant samples used in Run 1 were selected for this experiment (Suppl. material 1). The same DNA extracts obtained above were used to generate PCR amplicons targeting a longer fragment of the COI. PCR was performed using the Qiagen kit mentioned above. However, in order to target a larger fragment of the COI barcode region, the primer pair used for this experiment was LCO1490 (GGTCAACAAATCATAAAGATATTGG) – HCO2148 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al. 1994), isolating ~650 bp fragment. PCR amplification followed this cycle: 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 45 seconds, and a final extension phase at 72 °C for 7 minutes. Amplification was checked on 1% agarose gel and quantified using a QuBit.

The minion library preparation was performed following the manufacturer's instructions available on the Ligation Sequencing amplicons – Native Barcoding Kit 96 V14 (Available here: https://store.nanoporetech.com/native-barcoding-kit-96-v14.html). End-prep was performed using the NEBNext Ultra II End repair / dA-tailing Module (E7546, New England Biolabs, Massachusetts, USA), barcode ligation was performed using NEB Blunt/TA Ligase (M0367, New England Biolabs, Massachusetts, USA), and adapter ligation using the NEBNext Quick T4 DNA Ligase (E60567, New England Biolabs, Massachusetts, USA) and NEBNext Quick Ligation Reaction Buffer (B6058, New England Biolabs, Massachusetts, USA). The Barcode kit used was the Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies, United Kingdom). The flow cell used was an R10.4.1. flow cell (FLO-MIN114, Oxford Nanopore Technologies, United Kingdom). The library was loaded on a MinION Mk1C, which run for 72 hours. Sequence data (raw read signals) were basecalled using ONT's Guppy basecaller (version 6.5.7, super high accuracy model), and were demultiplexed using Guppy based on the barcode sequences used by the library preparation kit (NBD114.96). The database used for taxonomical assignment of these reads was generated using the COI sequences generated as outlined in the following paragraph.

Demultiplexed raw read data for the three Illumina MiSeq runs as well as for the Oxford Nanopore MinION run were uploaded on NCBI SRA under the BioProject number PRJNA1161788 titled "Ants metabarcoding for biosecurity".

Morphological examination and Sanger sequencing

The first 90 samples were morphologically examined to identify and count all individuals present in each sample. Samples varied from one single individual to 250 specimens, and from a single species to three. Morphological examination was conducted, and high-resolution images obtained using a Leica M205C stereo microscope with a Leica MC190 HD camera (Leica Camera, Germany).

Montage images were processed through the LAS X Life Science Microscope Software platform (ver. 5.2.0.26130).

Reference photos for each species identified in this study have been uploaded in the AntWeb with identification numbers ANTWEB1060404–ANTWEB1060449 (Table 2).

Species-level identifications were invariably based upon workers, with accompanying alates rare, and based on a combination of approaches, including examination of reference specimens and literature, depending on the requirements for the group (Suppl. material 1). Antwiki (http://Antwiki.org) was employed to ascertain the current status and access the literature for individual species. In some cases, unpublished keys, descriptions, and manuscripts were necessary. Clear consistency with available type specimens (see Suppl. material 1) and primary literature was always prioritised. When not written in English, and when no translation or translator was available, species descriptions were translated using translation software, including Google Translate, which provided an indication of content but was never the sole basis for species-level designation.

Ants preserved in the main Australian entomological collections were examined, either in person, or through the observation of high-resolution images provided by the institution. Abbreviations for collections and institutions from which material not publicly available on online platforms was examined are as follows:

AM Australian Museum, Sydney, Australia.
ANIC Australian National Insect Collection, Canberra, Australia.
MV Melbourne Museum, Melbourne, Australia.
NHMG Muséum d'histoire naturelle Genève, Genève, Switzerland.
NHMW Naturhistorisches Museum Wien, Vienna, Austria.
VAIC Victorian Agricultural Insect Collection, Bundoora, Australia.

Where morphological matches to types and descriptions were found, and no clear published evidence of an underlying species complex was found based on morphological differences, a species name was assigned. When a specimen could not be identified to species because of taxonomic uncertainty around the group, the 'species group' match is provided, along with a letter to designate separate morphospecies. The term 'group' is used here when there is evidence of known complexes under a single name, and the specimen exhibits some degree of difference from the type material. In cases where there were clear and consistent morphological differences between specimens that the literature designates a single species, the valid species group name is accompanied by morphospecies number (e.g., "sp. 1", "sp.2", etc). This was the case for undescribed morphological and molecular variation present in our specimens or convincingly demonstrated in the literature, suggesting a possible cryptic species, or simply an undescribed species. A representative specimen for each morphospecies was then databased and incorporated into the VAIC for possible use in future taxonomic work. Additionally, for each morphospecies, when more than one specimen was available, a series of "duplicate specimens" was lodged under the same VAIC code. Following morphological examination of all the specimens that generated the ASVs/morphospecies originally identified using MiSeq metabarcoding, 38 species could be identified and were selected for further sequencing (Table 2; Suppl. material 1).

DNA extractions from single ant specimens for Sanger sequencing were conducted on material that was previously non-destructively processed using the DNA extraction method outlined above. Single specimen DNA extraction was performed using either a partially destructive 5% Chelex protocol using a single ant leg (modified from Walsh et al. 1991), or a destructive Dneasy Blood and Tissue kit (Qiagen). PCR amplification targeted fragments of the 5' region of the COI locus using the primer pairs LCO1490-HCO2198 (Folmer et al. 1994). We used the primer pair fwhF2-fwhR2n (Vamos et al. 2017), for an additional two samples of *Iridomyrmex suchieri*, for which the longer fragment of COI failed amplification. The thermal cycling conditions consisted of: 2 minutes at 94 °C, 35 cycles of at 94 °C for 30 seconds, 51 °C for 45 seconds and 72 °C for 45 seconds, and a final extension of 2 minutes at 72 °C. PCR amplicons were sequenced using both forward and reverse primers by Macrogen Inc (Seoul, Korea). Consensus DNA barcode sequences were generated and aligned using the software MEGA 11 (Tamura et al. 2021), and a COI gene tree was generated using the Neighbor Joining algorithm and the Kimura 2 parameters model (Kimura 1980), with 5000 bootstrap replicates. The purpose of this tree was to compare the similarity of the different ant taxa and to visually examine closely related sequences. A total of 46 COI sequences were uploaded on GenBank with accession numbers PP600694–PP600739 (Table 2).

Results

Non-destructive DNA extractions generated successful amplification for a total of 176 of the 180 samples (Suppl. material 1). Of the 180 initial samples, the first 90 (Run1) were used for the comparison across platforms highlighted below, while the remaining 90 samples (Run2) were used only to provide an additional volume of samples of similar composition for the combined run (Run3). Therefore, only samples 1–90 were used for the morphological examination and the MiSeq/MinION experiment.

Comparison between MiSeq and MinION metabarcoding sequencing

The combined three MiSeq metabarcoding runs generated a total of 27,189,475 reads post quality control (Table 1). These belonged to a total of 64 ASVs/morphospecies from at least 15 genera (Suppl. material 1). Run1 generated 8,052,623 reads, Run2 generated 10,286,245 reads while the combined run, Run3, generated 8,850,607 reads (Table 1). When considering the reads from the samples included in Runs 1 and 2 in the combined run, these obtained 4,030,514 and 4,820,093 reads respectively, corresponding to 49.95% and 53.14% of the reads obtained in their respective single runs (Table 1). As part of the quality control, the negative control samples were checked for contaminations and reads from two taxa were recorded. As a consequence, reads from these two taxa were considered true positives only when representing the only ASV present in the sample or when present at high levels (>50% relative abundance). Overall, when comparing the results obtained in Run1 and Run2 with those obtained from Run3, all identifications consistently matched (100%), highlighting how no samples were lost due the lower number of reads per sample obtained in Run3 (Table 1).

Table 1. Number of total reads and per-sample reads obtained for each metabarcoding run. Individual MiSeq runs are reported in light blue, the combined MiSeq run is reported in dark blue and the MinION run is reported in green. Reads percentage difference are calculated compared with the single MiSeq run.

Run ID	Reads from single MiSeq run		Reads from combined MiSeq run		Reads from MinION	
	Total	Per sample	Total	Per sample	Total	Per sample
Run1 (Samples 1–90)	8,052,623	89,473.58	4,030,514 **(-49.95%)**	44,783.49	15,689,917 **(+94.84%)**	174,332.41
Run2 (Samples 91–180)	10,286,245	114,291.61	4,820,093 **(-53.14%)**	53,556.59		
Run3 (Samples 1–180)	8,850,607	49,170.04				

A total of 19,043,746 raw reads was obtained from the 72 h MinION run, across 88 samples (two samples failed to sequence, likely due to library preparation issues, Fig. 3). Of these, a total of 15,689,917 passed quality control and were mapped to one of the curated COI sequences generated combining the MiSeq data and the morphological examination (Tables 2, 3). This suggests that when considering the same number of samples, the 96-barcode kit used on the MinION can be considered equivalent to two times the read obtained on a V3 run on MiSeq, showing almost twice the number of reads (Table 1).

Overall, the results obtained using MinION matched those obtained using MiSeq (Suppl. material 1). Any incongruence between MiSeq and MinION results appear to be at the level of taxonomic assignment (due to the different database used). In fact, while the taxonomical identification may vary (Suppl. material 1), the results are consistent across the two platforms showing the same COI fragments (ASVs) being sequenced across the two experiments.

Metabarcoding using a publicly available reference database

For most of the 176 samples that generated results, a single ant taxon was recorded. However, a total of 20 samples showed more than one species reported both by the border identification and by the MiSeq identification (Suppl. material 1).

When comparing the MiSeq metabarcoding results with the preliminary morphological border identification, these matched or partially matched for the vast majority of samples (169 out of 212 combinations, 79.7%). We considered a match when both results showed the same species-level identification (N = 49, 23.1%; Suppl. material 1) and a partial match when both results matched at genus or family level without disagreeing at species level (e.g. *Iridomyrmex* sp. – *Iridomyrmex anceps*; N = 120, 56.6%; Suppl. material 1).

On the other hand, the remaining 43 combinations (20.3%; Suppl. material 1) were in disagreement by either reporting different identifications at the species or genus name (N = 29, 13.7%), or by missing/adding the record of a species that was present/absent (N = 14, 6.6%) (Suppl. material 1). Of the mismatching combination, a number was consistent across the dataset, suggesting the issue could be due to taxonomical assignment (i.e., matching records on reference database) as opposed to incorrect morphological identification from biosecurity taxonomist. For example, the species morphologically identified as *Rhytidoponera victoriae* was consistently identified as "*Melophorus* sp. 01" using MiSeq metabarcoding (N = 8).

Across all 176 samples, comparison of the short fragments of COI locus obtained here enabled identification of 64 different "operational taxonomic units" (OTUs) showing > 3% genetic variation. These morphospecies informed further steps for additional analyses.

Morphological examination, generation of COI sequences and curated database

For each OTU identified using MiSeq metabarcoding (Suppl. material 1), voucher specimens were identified from the bulk samples, photographed and sequencing of a longer fragment of COI was attempted. High-resolution photographs and morphological examination of specimens post-DNA extractions highlighted how morphological characters are retained even post molecular analysis as in the case of specimens of *Rhytidoponera metallica* in good condition, with most setae preserved (Fig. 1). Furthermore, important diagnostic features on head/frons and antennal scrobe (especially important for *Tetramorium*) can be observed unobstructed (Fig. 2). Small sub-erect setae on the scape, intact in *Myrmecia nigriceps* (Fig. 2), is an important trait to separate the species from close relatives for this species. In *Melophorus perthensis* (Fig. 2), the propodeum proportions are clear, the standing setae, or evidence of where standing setae should be, are clear. Important diagnostic features on head/frons and antennal scrobe can also be observed for *Iridomyrmex suchieri* (Fig. 2). The specimens representing the OTUs identified using metabarcoding were examined morphologically, aided by high resolution photographs. These morphological examinations enabled confirmation/dismissal of the species identity of each OTU, and enabled an enhanced focus on targeting each morphospecies for additional COI barcoding analysis. As a result, after morphological examination, a total of 38 morphospecies were confirmed from the 64 original OTUs. Of these, 46 COI sequences were successfully generated from 37 species and were submitted to GenBank, with accession numbers PP600694–PP600739 (Table 2). One morphospecies failed to generate a viable COI sequence and was morphologically destroyed in the DNA extraction process (Table 2).

Tetramorium caldarium was incorrectly identified morphologically as another *Tetramorium* species by biosecurity diagnosticians and an Australian species in the *Cardiocondyla nuda* group was misidentified as an exotic species (*C. minutior*) by a reference barcode (Suppl. material 1). Species belonging to the genus *Iridomyrmex* are usually identified to genus level by biosecurity diagnosticians since they don't pose biosecurity risks, and this meant they lacked verified reference barcodes (Suppl. material 1). There was sufficient morphological and molecular variation to divide both *Iridomyrmex bicknelli* and *Iridomyrmex chasei* into two morphospecies, with both possibly representing multiple species under a single valid name. The available morphological and molecular data suggest that the *Melophorus* specimen in sample 83 (Suppl. material 1) is near *Melophorus marius* but varies from the available material and available barcode data for the species (Table 3; Heterick et al. 2017; B. Heterick pers. comm.), suggesting the species falls within the *Melophorus marius* group complex acknowledged in Heterick (2021).

Pheidole sp. 1 was consistent with molecular barcodes and specimens misidentified as *Pheidole proxima* (see discussion below). *Rhytidoponera victoriae* group was identical to type specimens of *Ectatomma metallicum modestum*

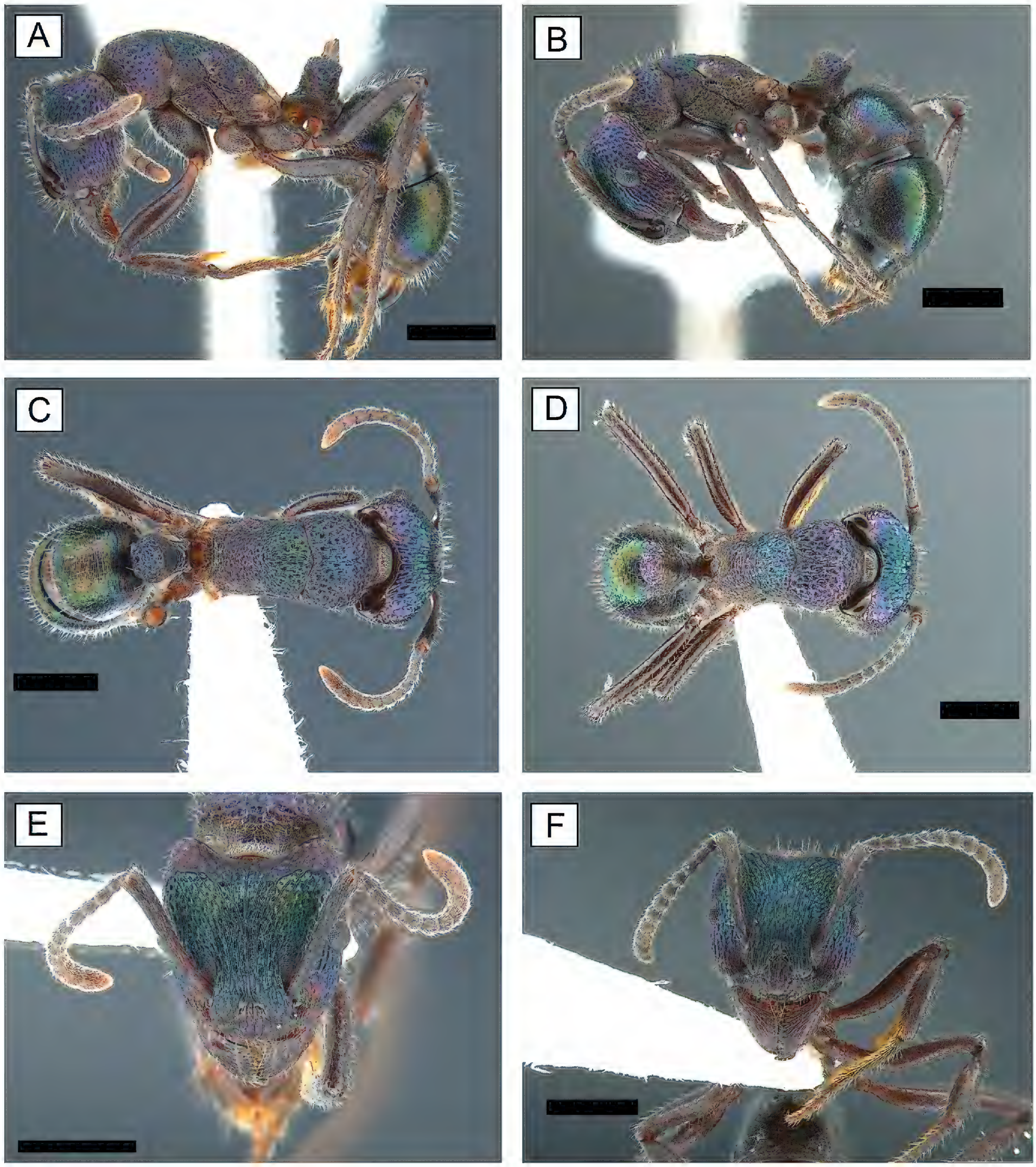

Figure 1. Two individuals of *Rhytidoponera metallica*, photographed post non-destructive DNA extraction. Lateral habitus (**A, B**), dorsal habitus (**C, D**) and details of the head (**E, F**). Scale bars: 1 mm.

Emery, 1895, currently synonymised with *Rhytidoponera victoriae* (André, 1896). However, it was both molecularly and morphologically distinct from *R. victoriae*. The three *Nylanderia* species were native but could not be convincingly matched to a described species. The Australian *Nylanderia* have not been monographed and exhibit considerable morphological overlap between species, with two of the three species from this study falling within dubious species groups (‘*braueri*’ and ‘*glabrior*’ groups, the other an intercaste), but not close enough to type material to confirm any species-level identification (Table 3).

Table 2. List of the 38 taxa identified using this integrative workflow. A total of 46 representative specimens, as well as duplicates, were deposited in the Victorian Agricultural Insect Collection (VAIC), with photos and metadata uploaded on AntWeb (AntWeb ID reported here). These belong to 17 different genera and 37 species. For each of the voucher specimens, a COI sequence was generated in this study and uploaded on GenBank, with accession number reported here.

Genus	Species	Specimen-Voucher	AntWeb ID	GenBank Acc. Number
Brachyponera	*Brachyponera lutea* (Mayr, 1862)	VAIC85547	ANTWEB1060419	PP600694
Camponotus	*Camponotus chalceus* Crawley, 1915	VAIC85545	ANTWEB1060417	PP600695
	Camponotus terebrans (Lowne, 1865)	VAIC85571	ANTWEB1060443	PP600696
Cardiocondyla	*Cardiocondyla nuda* group	VAIC85574	ANTWEB1060446	PP600697
Crematogaster	*Crematogaster laeviceps* Smith, F., 1858	VAIC85575	ANTWEB1060447	PP600698
Dolichoderus	*Dolichoderus ypsilon* Forel, 1902	VAIC85576	ANTWEB1060448	PP600699
Iridomyrmex	*Iridomyrmex bicknelli* group sp1	VAIC85535	ANTWEB1060407	PP600700
	Iridomyrmex bicknelli group sp2	VAIC85537	ANTWEB1060409	PP600701
	Iridomyrmex brunneus Forel, 1902	VAIC85569	ANTWEB1060441	PP600702
	Iridomyrmex chasei group sp1	VAIC85532	ANTWEB1060404	PP600703
	Iridomyrmex chasei group sp2	VAIC85534	ANTWEB1060406	PP600704
	Iridomyrmex discors Forel, 1902	VAIC85567	ANTWEB1060439	PP600705
	Iridomyrmex mjobergi Forel, 1915	VAIC85570, VAIC85577	ANTWEB1060442, ANTWEB1060449	PP600706, PP600707
	Iridomyrmex purpureus (Smith, F., 1858)	VAIC85568	ANTWEB1060440	PP600708
	Iridomyrmex rufoniger (Lowne, 1865)	VAIC85556	ANTWEB1060428	PP600709
	Iridomyrmex suchieri Forel, 1907	VAIC85533, VAIC85546, VAIC85554	ANTWEB1060405, ANTWEB1060418, ANTWEB1060426	PP600710, PP600711, PP600712
Melophorus	*Melophorus marius* group	VAIC85565	ANTWEB1060437	PP600713
Monomorium	*Monomorium fieldi* Forel, 1910	VAIC85572	ANTWEB1060444	PP600714
	Monomorium sordidum Forel, 1902	VAIC85573	ANTWEB1060445	PP600715
Myrmecia	*Myrmecia ludlowi* Crawley, 1922	VAIC85544	ANTWEB1060416	PP600716
	Myrmecia nigriceps Mayr, 1862	VAIC85543	ANTWEB1060415	PP600717
Notoncus	*Notoncus capitatus* Forel, 1915	VAIC85563	ANTWEB1060435	PP600718
	Notoncus gilberti Forel, 1895	VAIC85536	ANTWEB1060408	PP600719
Nylanderia	*Nylanderia* sp1	VAIC85541	ANTWEB1060413	PP600720
	Nylanderia sp2	VAIC85561	ANTWEB1060433	PP600721
	Nylanderia sp3	VAIC85549	ANTWEB1060421	PP600722
Ochetellus	*Ochetellus glaber* (Mayr, 1862)	VAIC85553	ANTWEB1060425	PP600723
Paratrechina	*Paratrechina longicornis* (Latreille, 1802)	VAIC85552	ANTWEB1060424	PP600724
Pheidole	*Pheidole bos* group	VAIC85555, VAIC85562	ANTWEB1060427, ANTWEB1060434	PP600725, PP600726
	Pheidole megacephala (Fabricius, 1793)	VAIC85558, VAIC85559	ANTWEB1060430, ANTWEB1060431	PP600727, PP600728
	Pheidole sp1	VAIC85557, VAIC85564	ANTWEB1060429, ANTWEB1060436	PP600729, PP600730
	Pheidole vigilans (Smith, F., 1858)	VAIC85540	ANTWEB1060412	PP600731
Polyrhachis	*Polyrhachis ammon* (Fabricius, 1775)	VAIC85539	ANTWEB1060411	PP600732
	Polyrhachis hookeri Lowne, 1865	VAIC85542	ANTWEB1060414	PP600733
Rhytidoponera	*Rhytidoponera metallica* (Smith, F., 1858)	VAIC85548, VAIC85551	ANTWEB1060420, ANTWEB1060423	PP600734, PP600735
	Rhytidoponera victoriae group	VAIC85538, VAIC85560, VAIC85566	ANTWEB1060410, ANTWEB1060432, ANTWEB1060438	PP600736, PP600737, PP600738
Solenopsis	*Solenopsis clarki* Crawley, 1922	-specimen destroyed-	N/A	N/A
Tetramorium	*Tetramorium caldarium* (Roger, 1857)	VAIC85550	ANTWEB1060422	PP600739

Table 3. Materials and references examined during the morphological identification process for each species. Material examined includes specimens examined from the Australian Museum (AM), Australian National Insect Collection (ANIC), Musee d'Histoire Naturelle Genève. (NHMG), Naturhistorisches Museum Wien, Vienna (NHMW), Museum Victoria (MV), Victorian Agricultural Insect Collection (VAIC). Type material (status determined from institution or associated specimen labels) can represent important related species or subspecies as well as the final species ID designation. The accession numbers from specific collections, or the unique specimen codes from AntWeb (https://www.antweb.org/). The references included here are reported at the end of the document.

ID	Material examined	Reference
Iridomyrmex chasei	**Physical:** VAIC:085654–085657; MV: HYM 46207. **Digital:** ANIC32000054 (type), CASENT0909508 (type), CASENT0909509 (type), FOCOL0022 (type).	Forel 1902, 1907; Wheeler 1934; Heterick and Shattuck 2011; Heterick 2021, 2022.
Rhytidoponera metallica	**Physical:** VAIC: 035569, 081181-2, 085658; MV: HYM 45408, HYM 45413, HYM 45411, HYM 45407, HYM 45693, HYM 45695, HYM 45692, HYM 45697, HYM 45698, HYM 45691, HYM 45585. **Digital** CASENT0900527, CASENT0915139, CASENT0907159, CASENT0915140.	Smith 1858; Brown 1958; Reichel 2003; Heterick 2021, 2022.
Iridomyrmex suchieri	**Physical:** VAIC: 081205, MV: HYM 48678. **Digital:** ANIC32017917 (lectotype), CASENT0909560, CASENT0909559 (type), CASENT0905048 (type).	Forel 1907; Crawley 1921; Heterick and Shattuck 2011; Heterick 2021, 2022.
Paratrechina longicornis	**Physical**: VAIC: 075553, 081370, 081371, MV: HYM 48386, HYM 48400, HYM 48388. **Digital:** CASENT0171035 ("type", type status dubious), CASENT0171073 ("type", type status dubious).	Latreille 1802; Forel 1901; LaPolla et al. 2013.
Ochetellus glaber	**Physical:** VAIC: 082694; MV: HYM 48308, HYM 48312; HYM 48324, HYM 48326. **Digital:** CASENT0909545 (type), FOCOL2839 (type).	Mayr 1862; Emery 1914; Viehmeyer 1914; Shattuck 1992; Heterick 2021, 2022.
Tetramorium caldarium	**Digital:** CASENT0102333 (type), CASENT0003150 (type), CASENT0249091 (type), worker, Lau, Latei Tonga, Fiji. BPBM; CASENT0915081.	Roger 1857; Agavekar et al. 2017; Heterick 2021, 2022.
Iridomyrmex bicknelli	**Physical:** VAIC: 074202, 082693, 085649, MV; HYM 48909, HYM 47632, HYM 47639; HYM 47644. **Digital:** CASENT0903081 (type), CASENT0909505 (type), CASENT0905046 (type).	Emery 1898; Forel 1902; Heterick and Shattuck 2011; Heterick 2021, 2022; Shattuck (unpublished).
Notoncus gilberti	**Physical:** MV: HYM 48141, HYM 48149. **Digital:** CASENT0909837 (Syntype), CASENT0909838 (type), FOCOL2219 (type).	Forel 1895; Wheeler 1934; Brown 1955; Taylor 1992; Heterick 2021, 2022.
Rhytidoponera victoriae group *[R. 'modesta']*	**Physical:** VAIC: 074631, 82128; MV: HYM 45689, HYM 45687, HYM 45689, HYM 45684, T 11355. **Digital:** AM: MFI_K340965, MFI_K340961Z; CASENT0907164 (syntype), CASENT0907166 (syntype), CASENT0907167 (type), CASENT0903829 (type).	Emery 1895; André 1896; Brown 1958; Reichel 2003
Polyrhachis ammon	**Physical:** VAIC: 081197, MV: HYM 47723. **Digital:** CASENT0910801 (type).	Fabricius 1775; Kohout 2013; Hoffmann 2015; Heterick 2021, 2022.
Pheidole bos group	**Physical:** MV: T 11416, HYM 46135, HYM 46134, HYM 46113, HYM 46138. **Digital:** ANTWEB1008221 (minor, type), CASENT0908012 (minor, type), CASENT0901546 (major, type), CASENT0908011 (major, type), CASENT0908029 (major, type), CASENT0908034 (minor, type).	Forel 1893, 1902, 1910, 1915; Crawley 1922; Clark 1938; Heterick 2021, 2022.
Iridomyrmex rufoniger	**Physical:** VAIC: 085650–085653, 081200–081203, 081382, MV: HYM 48613, HYM 48615, HYM 48607. **Digital:** CASENT0909555 (type), CASENT0909556 (type).	Lowne 1865b, Forel 1902; Heterick and Shattuck 2011; Heterick 2021, 2022.
Pheidole sp.1	**Physical:** MV: HYM 46164, HYM 46163, HYM 46165. **Digital:** FOCOL1419 (major, type), CASENT0919760 (major, type), NHMW: NHMW-HYM #2239–2240, 22241–22245 (type majors/minors collection), MNHG: 250430–250431, 250417 250432.	Mayr 1876; Forel 1902, 1910; Green et al. 2006; Sarnat et al. 2015; Heterick 2021.
Pheidole megacephala	**Physical:** VAIC: 59083, 081324. **Digital:** CASENT0104990 (major, type), CASENT0056016 (minor, type), CASENT0901410 (minor, type).	Forel 1913a; Eguchi 2001; Wilson 2003; Eguchi 2008; Fischer and Fisher 2013; Heterick 2021, 2022; Zhong 2021.

ID	Material examined	Reference
Nylanderia sp. 2 [*braueri* group]	**Physical:** ANIC: 32–035617, 32–035626, 32–060600, 32–066528, 32–035398, 32- 023493, 32–035418, 32–023036, 32–041494, 32–035726, 32–035727, 32–035732, 32–023582, 32–035395, 32–031578, 32–031592, 32–023519, 32–035592, 32–035593, 32–035484, 32–035698, 32–035691, 32–035917, 32–011707, 32–036922, 32–036907, 32–036912, 32–036861, 32–044552, 32–051371, 32–051458, 32–051422, 32–051425, 32–014040, 32–036880, 32–036881, 32–060603, 32–014039, 32–053585, 32–053599, 32–035899, 32–035896, 32–035979, 32–035980, 32–023186, 32–035877; MV: HYM48406, HYM48408, HYM48410, HYM48411. **Digital:** CASENT0911024 (type), CASENT0915719 (type), CASENT0903129 (type), CASENT0911015 (type), CASENT0911022 (type), CASENT0915721 (type), CASENT0903128 (type), CASENT0911014 (type), CASENT0917310 (type).	Shattuck (unpublished), Heterick 2021, 2022; Forel 1902, 1908, 1913b; Mayr 1868.
Pheidole vigilans	**Physical:** VAIC: 081472, 082133, 082136; MV: HYM 46200. **Digital:** CASENT0908016 (minor, type), CASENT0908018 (minor, type), CASENT0901543 (major, type), CASENT0908015 (major, type), CASENT0915458 (major, type).	Smith 1858; Brown 1971; Sarnat et al. 2015; Heterick 2021, 2022.
Nylanderia [*glabrior* group]	**Physical:** ANIC: 32-035617, 32-035626, 32-060600, 32-066528, 32-035398, 32- 023493, 32-035418, 32-023036, 32-041494, 32-035726, 32-035727, 32-035732, 32-023582, 32-035395, 32-031578, 32-031592, 32-023519, 32-035592, 32-035593, 32-035484, 32-035698, 32-035691, 32-035917, 32-011707, 32-036922, 32-036907, 32-036912, 32-036861, 32-044552, 32-051371, 32-051458, 32-051422, 32-051425, 32-014040, 32-036880, 32-036881, 32-060603, 32-014039, 32-053585, 32-053599, 32-035899, 32-035896, 32-035979, 32-035980, 32-023186, 32-035877; MV: HYM48406, 48408, HYM48410, HYM48411. **Digital:** CASENT0911024 (type), CASENT0915719 (type), CASENT0903129 (type), CASENT0911015 (type), CASENT0911022 (type), CASENT0915721 (type), CASENT0903128 (type), CASENT0911014 (type), CASENT0917310 (type).	Shattuck (unpublished); Heterick 2021, 2022; Forel 1902, 1908, 1913b; Mayr 1868.
Polyrhachis hookeri	**Physical:** MV: HYM 47866. **Digital:** CASENT0910828 (type), CASENT0915615 (type).	Lowne 1865b; Kohout 1994, 2000, 2008.
Melophorus marius group	**Digital:** CASENT0280488, CASENT0280489	Forel 1910, Wheeler, 1935, Heterick et al. 2017, Heterick 2021, 2022.
Iridomyrmex discors	**Physical:** VAIC: 085643–085648, MV: HYM 48961. **Digital:** CASENT0909523 (type), CASENT0915580 (type).	Forel 1902; Shattuck 1996; Heterick and Shattuck 2011; Heterick 2021, 2022.
Iridomyrmex purpureus	**Physical:** VAIC: 079400, MV: HYM 48533; HYM 48540. **Digital:** CASENT0909519 (type), CASENT0172039 (type), CASENT0903079 (type).	Smith 1858; Shattuck 1993; Heterick and Shattuck 2011; Heterick 2021, 2022.
Melophorus chauliodon	**Digital:** ANIC32-900189-2 (major, type), ANIC32-900189-1 (minor, type), ANIC32-900067-1 (minor, type), ANIC32-900067-2 (minor, type).	Heterick et al. 2017; Heterick, 2021, 2022.
Iridomyrmex brunneus	**Physical:** MV: HYM 47657. **Digital:** ANIC32039031 (type), CASENT0907612 (type), CASENT0909533 (type).	Forel 1902; Heterick and Shattuck 2011; Heterick 2021, 2022.
Myrmecia nigriceps	**Physical:** MV: HYM 44465. **Digital:** CASENT0915833 (type).	Mayr 1862; Clark 1951; Ogata and Taylor 1991; Heterick 2021, 2022.
Myrmecia ludlowi	**Digital:** CASENT0902802 (type).	Crawley 1922a; Ogata and Taylor 1991; Heterick 2021, 2022.
Iridomyrmex mjobergi	**Digital:** CASENT0907614 (type), CASENT0909548 (type).	Forel 1915; Heterick and Shattuck 2011; Heterick 2021, 2022.
Lioponera clara (=Cerapachys princeps)	**Digital:** CASENT0902752 (type).	Clark 1930, 1934; Heterick 2021, 2022.
Brachyponera lutea	**Digital:** CASENT0902499 (type), CASENT0915668 (type).	Mayr 1862; Schmidt and Shattuck 2014; Heterick 2021, 2022.
Camponotus chalceus	**Digital:** CASENT0910366 (minor, type), CASENT0911753 (minor, type).	Crawley 1915; Heterick 2021, 2022.

ID	Material examined	Reference
Camponotus terebrans	**Physical:** MV: HYM 49447. **Digital:** CASENT0911963 (type), CASENT0903534 (type), CASENT0911964 (type).	Lowne 1865a; McArthur et al. 1998; Shattuck and McArthur 2002; Heterick 2021, 2022.
Monomorium fieldi	**Digital:** CASENT0902286 (type), CASENT0904586 (type), CASENT0908769 (type), CASENT0908773 (type), CASENT0913585 (type), CASENT0913861 (type).	Forel 1910; Sparks 2015; Heterick 2001, 2009; Sparks et al. 2019; Heterick 2021, 2022.
Monomorium sordidum	**Digital:** CASENT0905835 (type), CASENT0908685 (type), CASENT0908686 (type).	Forel 1902; Heterick 2009, 2021, 2022; Sparks 2015; Sparks et al. 2019.
Cardiocondyla nuda group	**Physical:** VAIC: 075536; MV: HYM 46885. **Digital:** CASENT0919732 (type), CASENT0901758 (type).	Mayr 1866; Seifert 2003, 2008, 2022, 2023; Seifert et al. 2017; Heterick 2021, 2022.
Melophorus perthensis	**Digital:** ANTWEB1038575 (type), MCZ-ENT00303602-2 (major, type).	Wheeler 1934; Heterick et al. 2017; Heterick 2021, 2022.
Crematogaster laeviceps	**Physical:** VAIC: 56610, 56610. **Digital:** CASENT0908598 (type), CASENT0908599 (type), CASENT0902135 (type).	Smith 1858; Heterick 2021, 2022.
Solenopsis clarki	**Physical:** MV: T 21909 (PARATYPE). **Digital:** CASENT0902365 (type), CASENT0902366 (type).	Crawley 1922b; Heterick, B. E. 2009, 2021, 2022.
Dolichoderus ypsilon	**Physical:** MV: HYM 47453, HYM 47457. **Digital:** CASENT0909477 (type), ANIC32-015061 (type).	Forel 1902; Shattuck and Marsden 2013; Heterick 2021, 2022.
Nylanderia sp.3	**Physical:** ANIC: 32-035617, 32-035626, 32-060600, 32-066528, 32-035398, 32- 023493, 32-035418, 32-023036, 32-041494, 32-035726, 32-035727, 32-035732, 32-023582, 32-035395, 32-031578, 32-031592, 32-023519, 32-035592, 32-035593, 32-035484, 32-035698, 32-035691,, 32-035917, 32-011707, 32-036922, 32-036907, 32-036912, 32-036861, 32-044552, 32-051371, 32-051458, 32-051422, 32-051425, 32-014040, 32-036880, 32-036881, 32-060603, 32-014039, 32-053585, 32-053599, 32-035899, 32-035896, 32-035979, 32-035980, 32-023186, 32-035877; MV: HYM48406, 48408, HYM48410, HYM48411. **Digital:** CASENT0911024 (type), CASENT0915719 (type), CASENT0903129 (type), CASENT0911015 (type), CASENT0911022 (type), CASENT0915721 (type), CASENT0903128 (type), CASENT0911014 (type), CASENT0917310 (type).	Shattuck (unpublished); Heterick 2021, 2022; Mayr 1868; Forel 1902, 1908, 1913b.

Metabarcoding using a curated reference database

The morphological identification and the generation of curated COI sequences identified by expert taxonomists enabled re-running of the metabarcoding analysis using the enhanced database. This allowed comparison of the taxonomic identification results obtained after the four main steps of this workflow: the preliminary border biosecurity ID, the metabarcoding ID generated using the publicly available database, the species-level morphological identification and the second round of metabarcoding analysis using the updated and curated database (Fig. 3).

The preliminary border biosecurity identification, which was mainly aimed at determining presence/absence of exotic species, reported 41 correct species-level (Fig. 3, in green) and 38 correct genus-level identifications (Fig. 3, in yellow), as well as 23 incorrect or missing identifications (Fig. 3, in red). The MiSeq metabarcoding, which relied on publicly available data for the taxonomic assignment, could confirm only 20 correct species-level (Fig. 3, in green) and 56 correct genus-level identifications (Fig. 3, in yellow), with 26 incorrect or missing identifications (Fig. 3, in red). On the other hand, the taxonomic morphological examination could provide a species-level identification for 98 samples (Fig. 3, in green), with only four samples that could be identified only at the genus level (Fig. 3, in yellow). This enabled generation of 46 curated COI

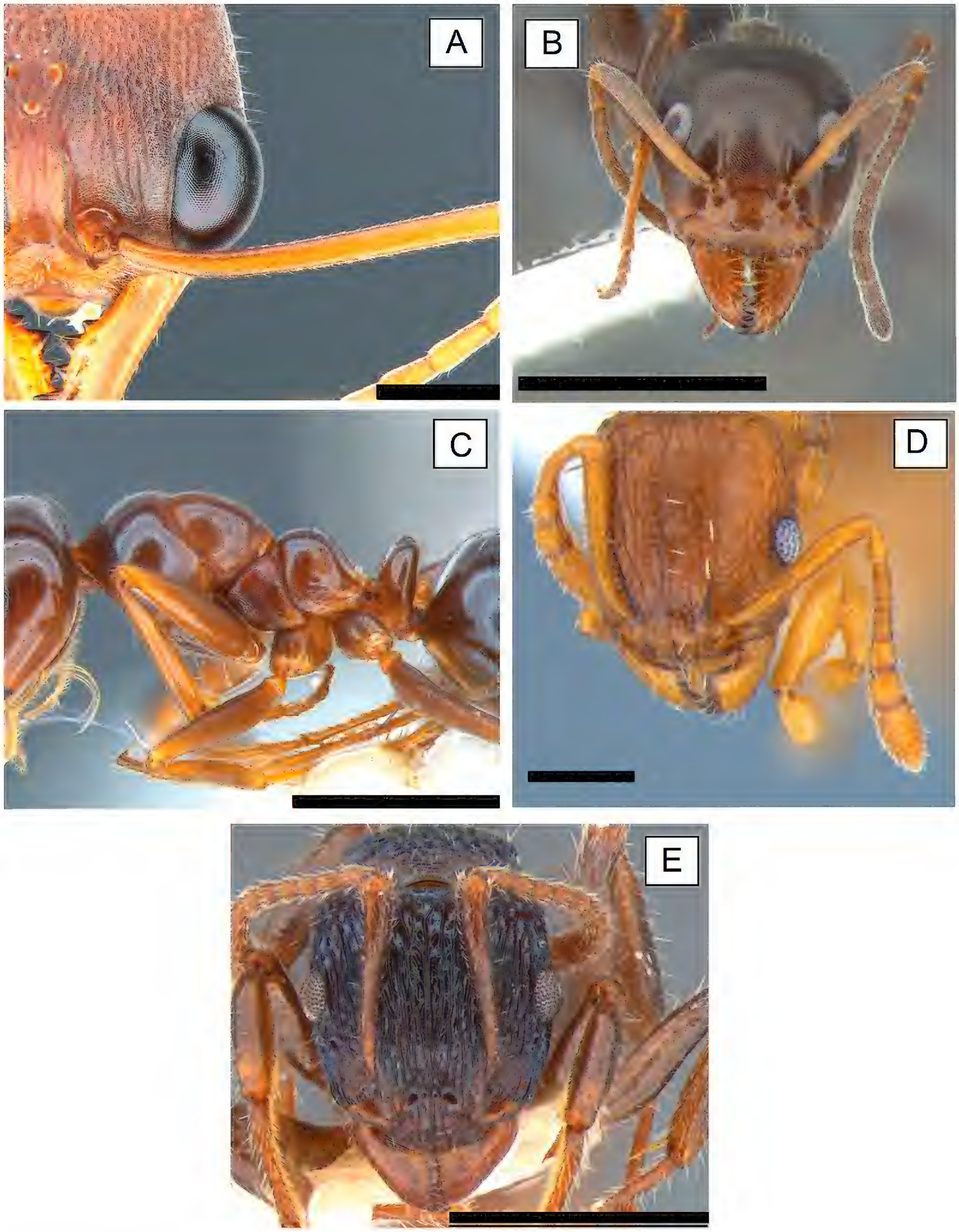

Figure 2. Examples of ant species photographed post non-destructive DNA extraction. Head of *Myrmecia nigriceps* (**A**), head of *Iridomyrmex suchieri* (**B**), thorax of *Melophorus perthensis* (**C**), head of *Tetramorium caldarium* (**D**), head of *Rhytidoponera victoriae* var *modestum* (**E**). Scale bars: 1 mm (**A–C, E**); 0.2 mm (**D**).

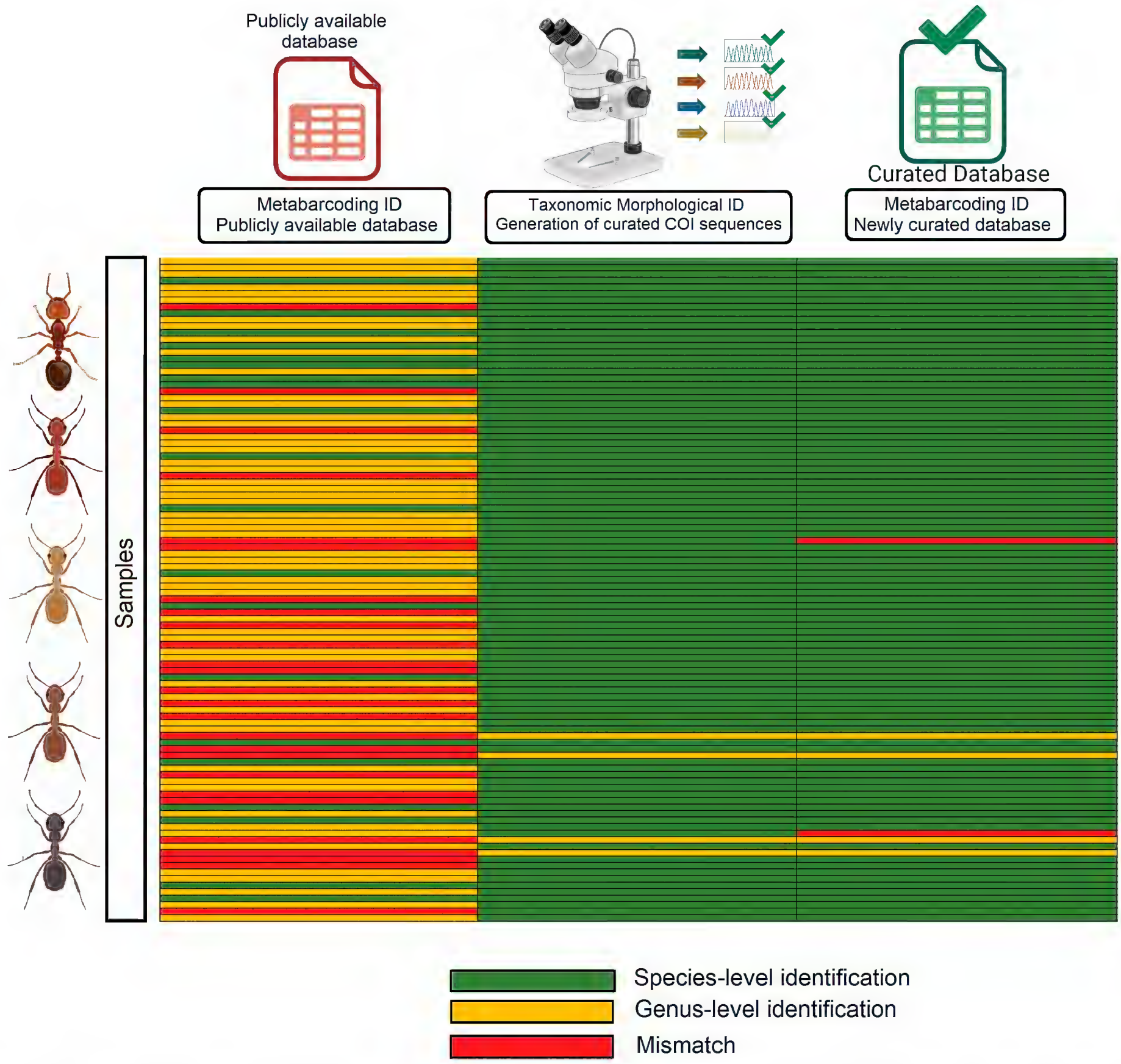

Figure 3. Increased species-level identification accuracy for the 90 samples processed using the workflow described in this work. The 90 samples were processed using MiSeq metabarcoding and a publicly available database. This highlighted a number of incorrect taxonomical assignments (in red). The morphological examination and resequencing of COI fragments from the voucher specimens preserved post non-destructive DNA extraction enabled provision of species-level identification for most of the samples (in green) and to use these curated sequences for the taxonomical assignment of the next metabarcoding analysis conducted using MinION. Parts of this figure were created with BioRender.com.

sequences that could then be used for the MinION sequencing and were compared to the previously generated MiSeq sequences. With the exception of two samples that failed to provide barcodes, the metabarcoding using the curated database could match the taxonomic identification, providing 96 species-level (Fig. 3, in green) and 4 genus-level identifications (Fig. 3, in yellow).

To confirm if this enhanced result was due to the improved taxonomic resolution of the curated database, the curated sequences generated were compared to the ASVs generated using MiSeq metabarcoding and found to match. This suggested that both MiSeq and MinION could sequence the same DNA and that the improved results are due to the curated database and not to the sequencing platform.

Discussion

A non-destructive metabarcoding workflow for the identification of ants collected during biosecurity surveillance programs

This work focused on Australian ants collected during biosecurity surveillance activities, highlighting the importance of these samples for biodiversity studies.

Australian ants represent a great model system, involving large numbers of bulk samples as well as numerous native/endemic species that are understudied and, therefore, underrepresented in public sequence database. These are very well-known issues linked with ant biodiversity, not only in Australia but worldwide (e.g., Ng'endo et al. 2013; Hanisch et al. 2017; Siddiqui et al. 2019; Schär et al. 2020).

Following the experiments conducted in our study, we propose an optimised workflow (Fig. 4) for the non-destructive high-throughput sequencing characterisation of ant samples collected during biosecurity surveillance activities. This allows faster and more accurate processing of biosecurity samples, while at the same time generating valuable data that can be used also for biodiversity assessments and studies.

To allow high-throughput processing of samples collected by biosecurity surveillance programs, we tested a non-destructive HTS approach comparing two different platforms to process large volumes of samples and determine the presence of "morphospecies" of interest. This step streamlines the first screening step allowing processing of hundreds of samples and immediate identification of priority pests, which are normally well-represented with DNA sequences available in public datasets. This fulfils the primary task of biosecurity activities, while also providing ASVs/OTUs for all other species present in the sample, including native and endemic species of ecological/taxonomical interest.

Ants collected during biosecurity surveillance activities using baited traps (Fig. 4A) should be processed using a non-destructive DNA extraction process (Fig. 4B) and can be sequenced using high throughput metabarcoding using either MinION or MiSeq platforms (Fig. 4C), as both these technologies generated sufficient sequencing depth for low-diversity samples, even when processing high volumes of samples.

Following sequencing, the generated ASVs or OTUs should be matched with publicly available data (Fig. 4D) to identify taxa of interest for biosecurity, as well as morphospecies of interest that should be processed further by expert taxonomists (4E). This process would allow quick identification of ant pest species of interest to biosecurity, distinguishing them from native species which may have no available reference sequence on a public database.

Non-target specimens could be of interest for biodiversity studies in case they provide novel genetic records for poorly characterised or undescribed species. For these reasons, these specimens should be databased and imaged to retain morphological voucher specimens (Fig. 4F) from which DNA re-extraction could be performed (Fig. 4G).

This would generate curated DNA sequences linked to a physical voucher specimen, improving publicly available database records and enabling correction/updating of records by allowing re-examination of a voucher specimen.

Ultimately, an additional advantage of this process would be its constant improvement in accuracy and turnaround times. Indeed, the more samples that

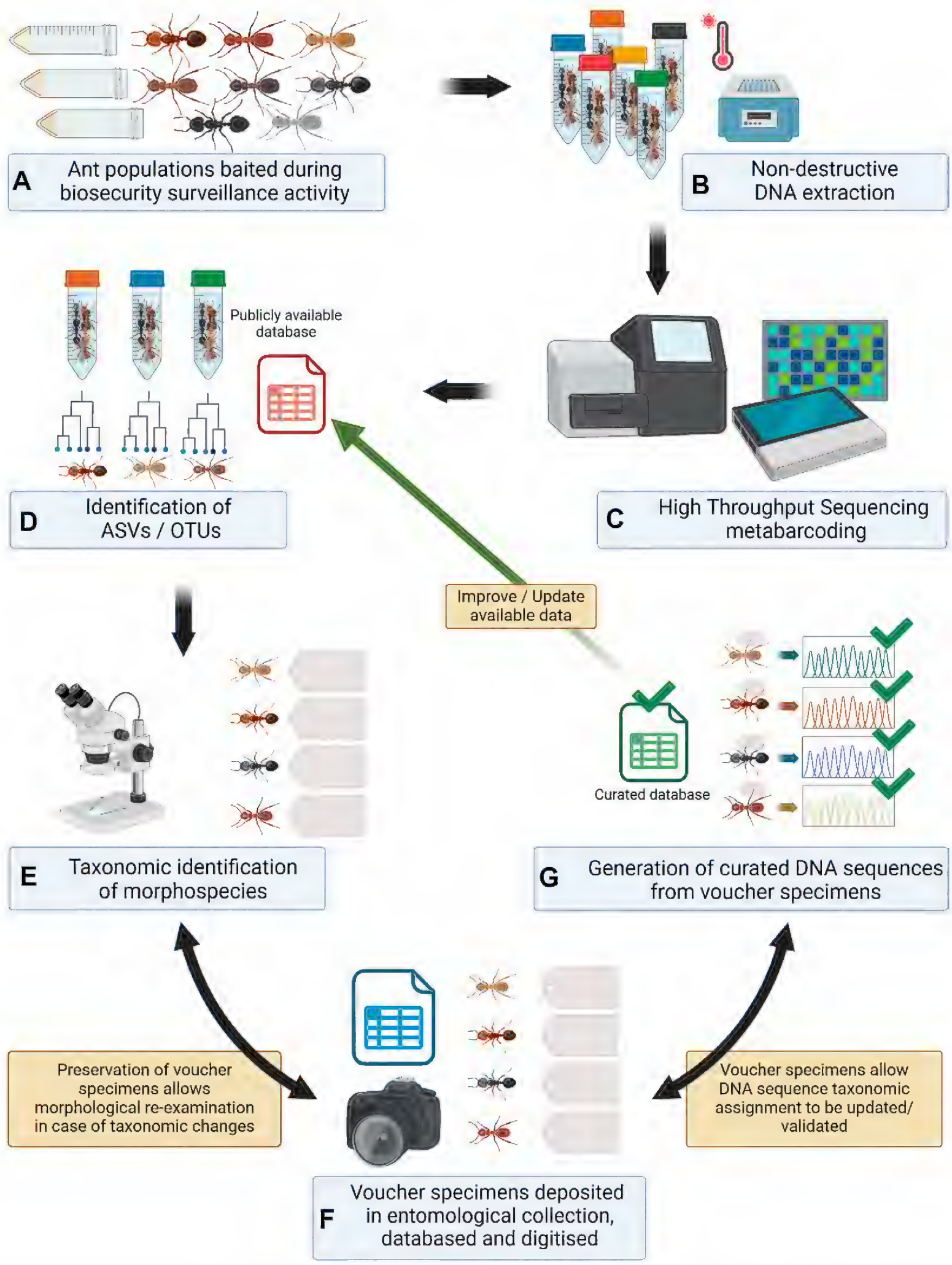

Figure 4. Proposed workflow for the high throughput processing of ants from biosecurity samples. Ant samples collected for biosecurity surveillance (**A**) are processed using a non-destructive DNA extraction (**B**) and sequenced using Illumina or Nanopore platforms (**C**), the generated ASVs/OTUs (**D**) are verified by examining the voucher specimens in order to identify morphospecies (**E**). Each morphospecies should be databased, deposited in a collection, and accompanied by high-resolution photographs (**F**), and a DNA sequence should be generated from each specimen (**G**) and uploaded into a public database in order to constantly improve the publicly available reference sequence data (Green arrow). This figure was created with BioRender.com.

are processed following this workflow, the more native species will be characterised and added to the public database, the more precise the first taxonomic assignment of ASVs/OTUs will be. In turn, this will require less and less morphological examinations, as more and more taxa will have a voucher specimen linked to a curated DNA reference.

Combining morphology and metabarcoding for ant identification benefits both biosecurity and biodiversity assessments

Integrating morphological examination, aided by high-resolution photographs, together with DNA sequences is a well-established procedure within integrative taxonomy (e.g., Padial et al. 2010; Martoni et al. 2021b). Here, integrating morphological and molecular data provided an additional layer of confidence in determining the number of species recorded during this work, even in those cases when the species have not been formally described, yet. Additionally, the experiments conducted here showed that the ant specimens post DNA extraction retained most of their taxonomically important characters, suggesting that a non-destructive DNA extraction process for ants can allow generation of curated DNA sequences while still retaining vouchers for entomological collections. The generation of curated sequences from specimens deemed "not of biosecurity concern" provides a much-needed insight into the genetic diversity of the Australian ant fauna, improving future molecular-based identification of native taxa.

Simultaneously curating a DNA database for the Australian ants also improves future biosecurity applications in case closely related exotic species find their way into the country. In the current study three previously introduced species with a limited established range in Australia were detected: *Paratrechina longicornis*, *Pheidole megacephala* and *Tetramorium caldarium* (Wetterer 2008, 2012; Wetterer and Hita Garcia 2015).

Misidentifications both through molecular and preliminary morphological identifications for groups of importance to biosecurity (e.g., *Tetramorium* and *Cardiocondyla*) and common native species (*Rhytidoponera victoriae* group, *Myrmecia*, and mid-sized *Iridomyrmex*) confirm that validating suspect specimen identifications with experts remains vital, as does the introduction of a standardised approach to generating barcode data and retaining specimens for future identifications.

Further, the taxonomic uncertainty present in several groups in this study, including genera like *Nylanderia* that are important to distinguish from related tramp species – organisms that have been spread globally by human activities – demonstrate the importance of preserving specimens for research and biosecurity studies for future validation when taxonomic work is eventually completed. *Myrmecia nigriceps* belongs to the taxonomically problematic *M. gulosa* group, with at least two molecular species existing under *M. nigriceps*, as recently demonstrated by Mera-Rodríguez et al. (2023). The specimen from this study, from Western Australia, closely matches the type material originally collected by Mayr, but Victorian-collected specimens present in other collections do demonstrate variation in COI barcodes and are morphologically consistent with Clark's *Myrmecia fasciata* Clark, 1951, synonymised with *M. nigriceps* (Ogata and Taylor 1991) but likely a distinct species.

Pheidole sp. 1 was consistent with molecular barcodes and specimens identified as *Pheidole proxima* Mayr, 1876, a native Australian species thought to be present in New Zealand (Green and Gunawardana 2006). This was suggested to be an incorrect record by Sarnat et al. (2015) and confirmed in this study by an examination of most of the existing type material for *P. proxima* and its subspecies (Forel collection), including minor workers from Mayr type series (details, including a revised distribution, Buxton *in prep*.).

Ectatomma metallicum modestum Emery, 1895 was determined to be a synonym of *Rhytidoponera victoriae* (André, 1896) but was later confirmed through an unpublished thesis (Reichel 2003) to be molecularly (one nuclear gene, one mitochondrial gene) and morphologically (structural colour) distinct from that species. Here, we found further evidence of both a molecular and morphological distinction (including new traits beyond colour) between *R. 'modesta'* and its sibling species (details unpublished). Crozier et al. (1986) also suggested a molecular divide existed between Victorian and Queensland populations of victoriae group specimens but did not identify the material further. Preliminary morphological examinations of material from Queensland, Lord Howe Island and NSW suggest that there may be further cryptic species currently under *R. victoriae* that warrant a revision of the group.

It is clear that numerous ant groups likely contain several species under a single valid name. However, we have taken the approach to use current valid names as accepted by the taxonomic literature, whenever a species matched a description and there was a lack of taxonomic work to convincingly demonstrate that previous designations have failed to accommodate the variation present in the specimen. This may not necessarily ally with all previous species delimitation practices but seemed the most consistent way of standardising an approach to suit both practical biosecurity and biodiversity surveillance.

Whilst a relatively large degree of COI and morphological variation was observed for several groups, this is at most an indication of potentially promising future research directions, with further integrative taxonomic work, including an examination of the wealth of material synonymised under current valid names, required to determine whether variability extends beyond what would be expected for a species.

Comparisons between different DNA sequencing platforms

Both short-read and long-read sequencing can be extremely valuable techniques, with sequence platform preference generally differing depending on the focus of the study, with longer reads generally being associated with better genetic resolution, especially useful in the case of cryptic species and species complexes that cannot be differentiated using shorter reads (Piper et al. 2019). In the past Oxford Nanopore Technologies (ONT) have been associated with long-read sequencing, leaving the field of short-read sequencing to Illumina. However, increasing improvements of ONT error rates (Morrison et al. 2020; Srivathsan et al. 2021) are shifting the short-read sequencing scene towards an increased use of MinION for metabarcoding, aided by the fact it doesn't necessitate the same large technological investments required by MiSeq (Piper et al. 2019).

The second aim of this work was to upscale the sample volume when using a MiSeq platform. Here we achieved processing of greater numbers of samples as a batch on the same run compared to similar insect bulk samples previously processed on a MiSeq flow cell (N = 47; Martoni et al. 2023 a,b). We increased sample volumes by doubling (N = 90, Run1 and Run2) and quadrupling (N = 180, Run3) the number of samples in each batch, enabling a higher throughput of data and a cheaper cost per sample.

As one would expect based on the compositional nature of HTS metabarcoding (Gloor et al. 2017), by combining two 90-sample libraries into a single

180-sample one, these ended up with approximately half the reads obtained in their respective single runs, with a decrease in the number of reads between 49.95% and 53.14% (Table 1). However, despite the lower number of DNA reads per sample, these were still abundantly sufficient to provide an identification with no species lost from Run3 due to insufficient number of reads. Additionally, all identifications obtained in the single runs (Run1 and Run2) consistently matched the identifications obtained in Run3 (100% match).

We demonstrated that sample volume upscaling from 90 samples to 180 did not impact the taxonomic identification of the ant species analysed in this work. Ultimately, increasing the number of samples that can be processed on a single MiSeq run not only makes the price-per-sample much cheaper, but it also enables a faster turn-around time between sample collection and data generation.

The third aim of this work was to analyse a similar volume of samples using Oxford Nanopore's MinION. In order to do this, we used the largest barcode kit commercially available, with the same DNA extracts used in a single MiSeq run. The two main advantages of using Oxford Nanopore technology to move from short-read to long-read sequencing are the increased genetic information that can be recovered by obtaining a longer DNA sequence, and the fact that the MinION platform can be used in relatively simple laboratories, being portable and in-field compatible, and not requiring many thousands of dollars of investment, enabling its use outside larger laboratories. When using long-read metabarcoding, the analysis conducted here showed that the 96-barcode kit provided by Oxford Nanopore is a valid tool to process at least 90 samples, and generated almost double the number of reads obtained when processing the same number of samples on the MiSeq. This suggests that the MinION platform may actually be capable of processing twice as many samples (N = 180) although this experiment could not be tested due to the lack of any commercially available kit providing more than 96 barcodes. Furthermore, the use of longer reads may prove especially useful in instances where low-diversity insect groups are observed, since a shorter barcode may not be able to separate closely related species, while a longer gene sequence might provide sufficient information to detect genetic diversity present. However, this was not the case for this work, where the shorter MiSeq barcode could successfully separate all species present in the bulk samples, as confirmed by the subsequent morphological examination.

One of the limitations of using the MinION platform has been the higher error rate compared to the Illumina platforms. Here, each sample processed using MinION recorded more than one species. This occurred in all MinION samples, even though many samples actually only consisted of a single species. However, the number of reads ascribed to each species was used as a clear marker to determine which record was a true positive and which record may be the result of barcode index switching errors. For example, for each sample the species with the highest number of reads generally outputted an average of ~170,000 reads, while the species with the second highest number of reads had an average of ~4855 reads. Relative abundance thresholds have been commonly adopted to mitigate the risk of false positive results due to low levels of contaminations and/or index switching (Piper et al. 2019). Further research is required to determine if a general threshold can be employed to determine when a record can safely be considered a true positive. Ideally, such a threshold should be based on relative abundance of reads, as it has already been widely discussed for MiSeq

metabarcoding (e.g., Martoni et al. 2022). Based on the results obtained here, a relative abundance threshold would be extremely effective on similar datasets, where the low diversity of the original sample allows for more stringent quality control and filtering steps without risking losing low-abundance taxa.

In general, the results obtained here are extremely promising. MinION-based long-read metabarcoding not only generated longer reads (~650 bp) but almost doubled the number of reads compared to a single run on the MiSeq. These results suggest that ant identification for biosecurity could be safely conducted using MinION platforms and future investments should be considered to research the development and application of dual unique indexes allowing processing of more samples on the same run. For example, doubling the number of samples (N = 180) would generate an average of approximately 87,000 reads per sample, very close to the average obtained using MiSeq. While increasing the number of samples of 50% (N = 135) would still generate approximately 115,000 reads per sample: an increase of 27% compared to the number of reads obtained with MiSeq.

Standardisation, and the importance of DNA sequence reference database curation to improve ant metabarcoding

The importance of comparability and standardization in high throughput sequencing workflows has been emphasized as critical for advancing biodiversity and biosecurity studies, a topic extensively addressed in recent literature (e.g., Piper et al. 2019; Lebas et al. 2022; Massart et al. 2022; Iwaszkiewicz-Eggebrecht et al. 2024). Certain aspects requiring standardization for broader biodiversity and biosecurity initiatives are equally pertinent to metabarcoding studies of ants. These include essential elements such as minimum metadata requirements, curation of reference databases, and rigorous quality and standard controls, all of which have been extensively debated and should be applied to the methodologies presented here. However, when considering the use of baited traps for collecting ants in biosecurity applications, some adjustments are necessary to tailor the broader standards established for insect biodiversity studies to the specific nature of the samples used for ant metabarcoding identification. Consequently, specific standardization requirements must be carefully considered and delineated.

For instance, consensus regarding the selection of target genetic regions for amplification, as advocated by Piper et al. (2019), has been widely adopted, often recommending the use of multiple markers via multiplex PCR (Iwaszkiewicz-Eggebrecht et al. 2024). Nonetheless, such extensive marker multiplexing may not be a requisite for studies focused solely on a single insect family, such as Formicidae, where the COI locus has proven sufficiently discriminatory for species differentiation. Moreover, the ant specimens collected via baited traps for biosecurity assessments present relatively clean samples, predominantly consisting of ants without significant debris or contaminants that might otherwise interfere with PCR processes. Consequently, a COI-based approach for ant identification stands as the preferred standard, with this study demonstrating the effectiveness of even a ~200 bp fragment in distinguishing closely-related species.

The nature of the sample (e.g., number of individuals, collection method, preservative used) profoundly influences subsequent analytical outcomes. For instance, whereas previous studies have encountered challenges such as primer specificity issues or incomplete species records (Basset et al. 2020, 2022), this

study successfully documented all species present in each sample. This accomplishment can be attributed to several factors, including the targeted capture efficiency of baited traps predominantly collecting ants, typically capturing a limited number of species compared to less selective traps that collect hundreds of individuals from diverse species (Martoni et al. 2023 a,b). Furthermore, the adoption of an amplicon sequence variants (ASVs) approach rather than operational taxonomic units (OTUs) likely contributed to the comprehensive species recording observed here. The ASV methodology analyses individual DNA reads separately rather than clustering them based on similarity thresholds. While the ASV approach may pose challenges in broader biodiversity surveys by potentially generating numerous records, its suitability is advantageous in ant metabarcoding, particularly when baited traps yield a select few species per sample. The workflow detailed in this study benefited significantly from ASV utilization, facilitating the morphological examination of a larger number of specimens which were subsequently confirmed to belong to a limited set of morphospecies, underscoring the ASV-based approach's capability to discern genetic diversity even within species. This enhanced the likelihood of capturing the entire species composition within a sample and aiding subsequent morphological assessments.

The morphological identification aspect of this workflow is identified as a critical component necessitating standardized procedures. Morphological identification should not be subjective but grounded in thorough examination of scientific literature and voucher specimens housed in reference collections. Similar to other scientific activities, morphological identification must be replicable, with clear protocols detailing the steps leading to taxonomic assignments. Here, we have provided detailed methodologies for each morphological identification, including voucher specimen identification numbers and comprehensive literature citations outlining diagnostic criteria (Table 2).

Finally, the curation of DNA reference databases has been extensively debated in terms of standardization requirements, and warrants a more in-depth discussion here. DNA based methods aiming to provide a species-level taxonomical identification for insect groups rely entirely on reference databases and repositories linking genetic sequences to taxonomic names (Keck et al. 2023). The incompleteness of reference databases is an important argument to explain missed detections of invertebrate taxa when using metabarcoding (Dowle et al. 2016; Elbrecht et al. 2017; Watts et al. 2019). Such incompleteness can be attributed to several reasons, from a paucity of taxonomists and scarcity of funding for taxonomic work (Engel et al. 2021), to strong taxonomic and geographic biases in the origin of the sequences present in the database (Weigand et al. 2019; Keck et al. 2023). Additionally, other issues include, and are not limited to, mislabelled sequences, sequencing errors, sequence conflicts, taxonomic conflicts (Piper et al. 2019; Marques et al. 2021; Keck et al. 2023), as well as the so-called "dark taxa" that are present on the database but lack a taxonomic identification (Page 2016).

In regions with diverse and endemic biota, such as Australian ants, maintaining a well-curated reference database assumes heightened significance. Australian ants are characterized by substantial endemism, with many taxa exhibiting unique genetic profiles largely confined to the country, and the results obtained here could be ascribed to some of the issue mentioned above. For instance, some samples matching (100%) identical DNA sequences had conflicting taxonomic identifications, as in the case of *Iridomyrmex anceps* and *I.*

suchieri. This has been highlighted as "taxonomic mislabelling" by Keck and colleagues (2023), as identical sequences are erroneously labelled with different taxonomic names. Other sequences had no closely related record available to match with on the database and could only be identified to family level, as "Formicidae sp. 1". This is an example of "missing taxa" (Keck et al. 2023), as described species scientifically recognised that are not present in the database can constitute an important limitation. Another instance showed one of our specimens, *Iridomyrmex rufoniger*, matched (100%) a sequence already present on the database, but had only been identified to the genus level, *Iridomyrmex* sp. DQ249968.1, a perfect example of a dark taxon, which is present on the database but lacks precise taxonomic identification (Page 2016). Finally, multiple instances matched a sequence that was incorrectly identified as a different species, as in the case of *Rhytidoponera victoriae* matching a record labelled as *Melophorus* sp., representing instances of "taxonomic conflict" (Keck et al. 2023) where previous taxonomical misassignments have not been revised in the database.

A total of 46 new COI DNA barcode reference sequences were generated from previously uncharacterised/unidentified specimens. These sequences provide the first genetic information for a number of ant species for which molecular-based identification has so far been challenged by their absence on public databases. As a result, physical voucher specimens linked to DNA sequences are now preserved in entomological collections, enabling future morphological re-examination of the vouchers as well as future assessments and comparisons with novel samples.

Ultimately, we think that recent developments surrounding the accessibility and increased use of metabarcoding and metagenomic techniques, as well as their reliance on public reference databases, require a shift in our way of approaching the generation of DNA sequences, from "quantity" to "quality". With the costs of sequencing becoming more and more affordable, and the use of novel, high-throughput techniques (e.g., mega-barcoding; Srivathsan et al. 2021; Chua et al. 2023), it is increasingly clear that the impediment is not sequencing but obtaining taxonomically correct identification of sequenced specimens, which is provided thanks to highly skilled taxonomical expertise. Here, we provide a more standardised approach to the identification of Australian ants, demonstrating how to generate taxonomically accurate sequences that will improve molecular-based identification of taxa for biodiversity and biosecurity.

The workflow proposed in this study endeavours to link each genetic record with physical voucher specimens and high-resolution images, ensuring the veracity of taxonomic identifications derived from genetic material. While databases like BOLD (Ratnasingham and Hebert 2007) permit inclusion of photographic data, lower resolution whole specimen images may prove insufficient for accurate ant identification. Non-destructive or semi-destructive DNA extraction methods, particularly from leg segments, are recommended to preserve specimens for future taxonomic scrutiny. Moreover, studies generating novel DNA sequences should document the methodologies employed for taxonomic identification, akin to the protocols used for describing and diagnosing new taxa. Given the distinct biodiversity and endemism of ants, establishing a bespoke reference database for Australian species appears paramount, encompassing genetic sequences, links to voucher specimens, high-resolution morphological images, and detailed taxonomic attribution protocols. Such a

resource would be indispensable for managing the many currently undescribed species, utilizing genetic sequences as proxies for diversity akin to the Barcode Index Number (BIN) system within BOLD (Ratnasingham and Hebert 2007).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: MJB, KSS, FM. Data curation: FM, JB. Formal analysis: LR, JB, FM, TL, RLS. Funding acquisition: FM. Investigation: JB, FM. Methodology: LR, FM, RLS, JB, KSS, TL. Project administration: FM. Supervision: MJB, KSS. Visualization: FM. Writing - original draft: FM, MJB, JB. Writing - review and editing: JB, RLS, LR, KSS, FM, TL, MJB.

Author ORCIDs

Francesco Martoni https://orcid.org/0000-0001-8064-4460
James Buxton https://orcid.org/0000-0002-9309-6181
Kathryn S. Sparks https://orcid.org/0009-0003-4094-1139
Tongda Li https://orcid.org/0000-0001-9430-2779
Reannon L. Smith https://orcid.org/0000-0002-3794-5900
Lea Rako https://orcid.org/0000-0002-3528-8978
Mark J. Blacket https://orcid.org/0000-0001-7864-5712

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information. COI sequences are available on GenBank with accession numbers PP600694–PP600739. Reference photos are available on AntWeb with identification numbers ANTWEB1060404–ANTWEB1060449. Metabarcoding raw read data is available on NCBI SRA under the BioProject number PRJNA1161788, titled “Ants metabarcoding for biosecurity”.

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Supplementary material 1

The 180 samples processed for this study, including their metadata

Authors: Francesco Martoni, James Buxton, Kathryn S. Sparks, Tongda Li, Reannon L. Smith, Lea Rako, Mark J. Blacket

Data type: docx

Explanation note: Morphological ID and MinION ID. For each sample, the morphological identification provided by border diagnosticians (Border ID) is reported together with the taxonomic identification matched after the MiSeq "Run1 and Run 2" for 90 samples (MiSeq 90 samples) and the MiSeq run for 180 samples (MiSeq 180 samples). Morphological identification provided by expert taxonomists (Morphological ID) was performed only on the first 90 samples and on any additional sample containing a unique ASV reported by the MiSeq metabarcoding. Morphological identification contributed to generate the curated COI sequences that were then used for the MinION experiment. MinION analysis was performed only on the first 90 samples.

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